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Department of Environmental Engineering

PhD Thesis
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Preface

This PhD thesis, entitled "Bioremediation, nutritional value and biorefining of macroalga *Saccharina latissima*", comprises the research carried out at the Department of Environmental Engineering, Technical University of Denmark from September 01, 2012 to October 31, 2015. Professor Irini Angelidaki and Assistant Professor Susan Løvstad Holdt were supervisor and co-supervisor, respectively.

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-IV**.

I Marinho GS, Holdt SL, Birkeland MJ, Angelidaki I (2015) Commercial cultivation and bioremediation potential of sugar kelp, *Saccharina latissima*, in Danish waters. Journal of Applied Phycology 27(5):1963-1973. DOI: 10.1007/s10811-014-0519-8

II Marinho GS, Holdt SL, Angelidaki I (2015) Seasonal variations in the amino acid profile and protein nutritional value of *Saccharina latissima* cultivated in a commercial IMTA system. Journal of Applied Phycology 27(5):1991-2000. DOI: 10.1007/s10811-015-0546-0

III Marinho GS, Holdt SL, Jacobsen C, Angelidaki I (2015). Lipids and composition of fatty acids of *Saccharina latissima* cultivated year-round in integrated multi-trophic aquaculture. Marine Drugs. 13(7):4357-74. DOI: 10.3390/md13074357

IV Marinho GS, Alvarado-Morales M, Angelidaki I (2015). Valorization of macroalga *Saccharina latissima* as novel feedstock for fermentation-based succinic acid production in a biorefinery approach and economic aspects. Submitted.

In this online version of the thesis, paper **I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljoevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

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Over the last three years I had a very good time at DTU Environment where I had the chance to develop myself both personally and professionally, also I had the opportunity to meet many great people that made my stay very enjoyable and to whom I'm grateful.

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Likewise I would like to thank my co-supervisor Assistant Professor Susan Løvstad Holdt without whom I would probably not have come to Denmark in the first place, and for hosting me at her place in the first weeks after my arrival-I won't forget that. Also for being always available when I needed to discuss ideas, results and drafts.

Additionally, I wish to thank to my colleague and friend Merlin Alvarado Morales, with whom I had the privilege to work in the final part of my PhD research project.

I would like to tank to Hjarnø Havbrug A/S for all logistic support in the "field workò, special thanks to Malene Mølgaard, Teis Boderskov, Peter Schmedes and Zydromas.

I would like to give thanks to: Ingólfur Bragi Gunnarsson, Morten Foldager Petersen, Jonathan Van Wagenen, Mike Podevin, Davide De Francisci, Hector Garcia and Daniel Moreira Fontes Lima.

At last I would like to thank my whole family who made me who I am. Special thanks are given to my beloved wife for she has always been there supporting and believing in me, and has joined me in many of the long trips to Horsens where I did the "field work".

Summary

Macroalgae have the ability to assimilate and convert waste nutrients (N and P) into valuable biomass. In this context, they have been extensively studied for their bioremediation potential for integrated multi-trophic aquaculture (IMTA). With a global aquaculture production of 23.8 million tonnes in 2012, macroalgae are a valuable source of vitamins, minerals, lipids, protein, and dietary fibres. Macroalgae have been used as food since ancient times in Asian countries, while in Europe they have lately been introduced as healthy food. Moreover, recently macroalgae have been receiving increasing attention as sustainable feedstock for biorefinery. Nevertheless, macroalgae resources are still very little explored in western countries.

The aim of this study was fulfilled by the investigation of the bioremediation potential of the macroalga *Saccharina latissima* cultivated at a reference site (control) and at an IMTA site during 12 months (May 2013-May 2014), and assessing the effect of cultivation site and harvest time. Moreover, a comprehensive chemical and nutritional characterization of the produced biomass was made, and its potential as food and/or feed discussed. Finally *S. latissima* biomass was tested as feedstock for fermentation-based succinic acid production in a biorefinery approach.

Maximum biomass yield over one growing season was achieved in August (1.08-1.51 kg fresh weight (FW) m^{-1} of cultivation line) and September (0.92-1.49 kg FW m^{-1}). Biomass yield directly correlated with the nutrient removal which similarly peaked in August (5.02-7.02 g N m^{-1} and 0.86-1.23 g P m^{-1}) and September (4.73-7.24 g N m^{-1} and 0.83-0.96 g P m^{-1}). Moreover, both biomass yield and nutrient removal were higher in the IMTA site compared to the reference site in August ($p<0.05$). Additionally, macroalgal cultivation over two growing seasons enhanced the biomass yield and thus value, but not the bioremediation capacity.

Harvest time had a significant impact in overall chemical composition, while cultivation site did not generally result in marked differences. The growth of epiphytic organisms from July to November makes the biomass unsuitable for human consumption, thus biomass meant to be used as food should be harvested in May. Protein content increased significantly from 1.3% dry matter (DM) in May to 10.8% DM in November. Similarly, the maximum essential amino acid (EAA) score was found in November (68.9%). Thus, results suggest an apparent mismatch between harvest time for human consumption (May) and the highest nutritional value of the protein in the biomass (November). The growth of epiphytes did not change the amino acid content or EAA score. However, the

protein content and composition did not comply with the requirements for standard protein ingredients for fish feed (i.e. fishmeal, soymeal). The lipid concentration varied from 0.62%–0.88% DM in July to 3.33%–3.35% DM in November ($p<0.05$). Polyunsaturated fatty acids (PUFA's) made up more than half of the fatty acids with a maximum in July (52.3%–54.0% fatty acid methyl esters). This including the most appreciated health beneficial PUFA's, eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), but also arachidonic (ARA) and stearidonic acid (SDA). Season of harvest is important for the choice of lipid quantity and quality, but the macroalga provides better sources of EPA, DHA and long-chain (LC)-PUFA's in general compared to traditional vegetables. Regarding safety regulations, however, the main conclusions on the mineral analyses showed that high concentrations of iodine (up to 5,001 mg kg^{-1} DM) in the biomass may be of concern for human consumption, while the concentrations of total arsenic (up to 63.3 mg kg^{-1} DM) may restrict utilization as ingredient for feed.

Seasonal variations in the content of carbohydrates, and fermentable sugars, had a significant impact on the succinic acid yield and titer. A maximum succinic acid yield of 91.9% (g g^{-1} of total sugars) corresponding to 70.5% of the theoretical maximum yield was achieved; while succinic acid titer amounted up to 36.8 g L^{-1} with maximum productivity of 3.9 g L^{-1} h^{-1}. The high content of total phenolic compounds in the macroalga (July-August: 5-1% DM), and high concentration of inorganic nutrients in the solid residue recovered after enzymatic hydrolysis, makes co-production of antioxidants (i.e. phenols) and fertilizer very attractive. This was demonstrated to have the potential to increase the cost-effectiveness of the biorefinery facility.

This study gives comprehensive information of the bioremediation potential of *S. latissima* cultivated commercially in the inner Danish waters. Year-round data show that harvest time can be effectively used to optimize the bioremediation capacity, and the biomass yield and application/value. The macroalga can be a source of valuable proteins, specific amino acids and food; however, high concentrations of iodine and total arsenic may be of concern regarding food and feed safety regulations, respectively. On the other hand, *S. latissima* is a promising feedstock for fermentation-based succinic acid production with co-production of phenols, and fertilizers.

Dansk sammenfatning

Makroalger kan optage og konvertere overskydende næringssalte (N og P) om til værdifuld biomasse. De har derfor været undersøgt i stor udstrækning for deres biofilter potentiale i integreret multi-trofisk akvakultur (IMTA). Makroalger er en værdifuld ressource såsom vitaminer, mineraler, lipider, proteiner og kostfibre, med en global akvakultur produktion på 23,8 mill ton i 2012. I Asien har man anvendt makroalger til konsum fra gammel tid, mens man først for nylig er blevet introduceret for makroalger, som en sund fødevare. Derudover har anvendelsen af makroalger, som råmaterialer i bioraffinaderi konceptet også fået opmærksomhed indenfor det sidste årti. Ikke desto mindre er makroalger som ressource meget lidt undersøgt i den vestlige verden.

Formålet med denne afhandling blev indfriet ved at undersøge biofilter potentialet i makroalgen *Saccharina latissima* dyrket ved et reference sted (kontrol) og en IMTA lokalitet over 12 måneder (maj 2013-maj 2014), og bedømme effekten af dyrkningssted og høsttid. Derudover, blev den producerede biomasse karakteriseret dybdegørende mht. den kemiske sammensætning og næringsværdi, og potentialet for anvendelsen som fødevare og/eller foder blev diskuteret. Endelig blev *S. latissima* testet som råmateriale i en fermenteringsproces, der kunne producere succinatsyre (ravsyre), som led i en bioraffineringsproces.

Henover den første vækstsæson blev det maksimale udbytte opnået i august måned (1,08-1,51 kg vådvægt (VV) m^{-1} af dyrkningsreb) og september (0,92-1,49 kg VV m^{-1}). Biomassens udbytte havde direkte sammenhæng med fjernelsen af næringssalte, der også havde højeste koncentration i august (5,02-7,02 g N m^{-1} og 0,86-1,23 g P m^{-1}) og september (4,73-7,24 g N m^{-1} og 0,83-0,96 g P m^{-1}). Derudover var biomasseudbyttet og næringssaltsfjernelsen højere ved IMTA lokaliteten sammenlignet med referencedyrkningsområdet i august (p<0,05). Ved at dyrke makroalgerne videre over to vækstsæsoner så øgedes udbyttet af biomasse og derved også værdi, men dog ikke biofilter kapaciteten.

Høsttidspunktet havde en signifikant effekt på den generelle kemiske sammensætning af biomassen, mens dyrkningsstederne generelt ikke påvirkede biomassen markant. Påvækst af epifytter fra juli til november gør biomassen uegnet til konsum, og skal derfor høstes i maj hvis det er anvendelsen. Proteinindholdet øgedes signifikant fra 1,3% tørvægt (TV) i maj op til 10,8% TV i november. Ligeledes findes den maksimale essentielle aminosyre score (EAA) i november (68,9%). Derved udviser resultaterne mangel på sammenfald imellem høsttid og egnethed til konsum (maj) og den højeste næringsværdi af protein i biomassen (november). Epifyt-påvæksten ændrede ikke

aminosyreindholdet og heller ikke EAA scoren, men proteinindholdet og -sammensætningen kunne ikke imødekomme de krav der er for standardproteiningredienser i fiskefoder (f.eks. fiskemel og sojabønner). Lipid-koncentrationen varierede fra 0,62%–0,88% TV i juli til 3,33%–3,35% TV i november (p<0,05). Flerumættede fedtsyrer (PUFAer) udgjorde op til over halvdelen af fedtsyrerne med et maksimum i juli (52,3%–54,0% fedtsyre methylestre). Disse PUFAer inkluderer syrerne eicosapentaenoic (EPA; 20:5n-3) og docosahexaenoic (DHA; 22:6n-3), der er de mest helsefremmende, men derudover også arakidonsyre (ARA) og stearidonic syre (SDA). Høstsæsonen er vigtig for hvilken lipid-kvantitet og -kvalitet, der ønskes, men disse marine grøntsager sikrer generelt en bedre kilde af EPA, DHA og de lang-kædede (LC)-PUFAer sammenlignet med traditionelle grøntsager. Ud fra mineralanalyserne er hovedkonklusionerne mht. fødevare- og foderlovgivningerne dog at den høje koncentration af jod (op til 5.001 mg kg^{-1} TV) kan være et problem mht. konsum, mens det er koncentrationen af total arsen (op til 63,3 mg kg^{-1} TV), der kan begrænse anvendelsen i foder.

Sæsonvariationen i indholdet af carbohydrater, og fermenterbare sukre, har en signifikant påvirkning på succinatsyre udbyttet og koncentration. Det maksimalt opnåede udbytte af succinatsyre var 91,9% (g g^{-1} af total sukker), hvilket svarer til 70,5% af det teorestiske maksimale udbytte, mens succinatsyre koncentrationen kom op på 36,8 g L^{-1} med en maksimal produktivitet på 3,9 g L^{-1} h^{-1}. Den høje koncentration af totale phenoliske indholdsstoffer i makroalgen (juli-august: 5-1% TV), og høje koncentrationer af uorganiske nærringssalte i det faste affaldsprodukt genfundet efter enzymatisk hydrolyse, gør sam-produktionen af antioxidanter (f.eks. phenoler) of gødning meget attraktiv. Dette var ydermere tydeliggjort ved at have potentiale i at øge omkostningseffektiviteten af en sådan bioraffinaderi facilitet.

Dette studie giver omfattende information om biofilterpotentialet i *Saccharina latissima* dyrket kommercielt i de indre danske farvande. Helårlige data viser at høsttid kan udnyttes effektivt for at optimere biofilter kapaciteten, biomasseudbyttet og anvendelsen/værdien. Makroalgen kan udgøre en kilde af værdifulde proteiner, specifikke aminosyrer og som fødevare; dog udgør de høje koncentrationer af jod og total arsen muligvis en bekymring mht. respektive fødevare- og foder sikkerhedslovgivninger. På den anden side så er *S. latissima* et lovende rå-materiale til fermenteringsbaseret succinatsyre produktion med sam-produktion af phenoler og gødning.

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1 Introduction

1.1 Background

World aquaculture's food fish supply has been developing at an average annual rate of 8.8 percent over the last three decades (1980-2010), and further development is expected. In 2010 this production sector reached a new historic maximum, supplying 47 percent of total fish consumed worldwide (FAO 2012). However, there is rising social and political concerns regarding the environmental impact of intensive monoculture production systems (e.g. finfish and shrimp). Open water aquaculture is restricted by an emission quota of N and P in Denmark. This has been major limiting factor for expansion of Danish aquaculture fish production, which has been capped for 20 years since 1987 (Holdt and Edwards 2014). In the Norwegian salmon aquaculture around 62-63% of feed-N and 70% of feed-P are released into the environment, both under the particulate and dissolved forms (Olsen et al. 2008; Wang et al. 2012). Dissolved inorganic nutrients account for approximately 45% and 18% of total produced N and P, respectively (Wang et al. 2012), which are directly available for algae and may lead to eutrophication (Nixon 1995; Skogen et al. 2009). In the light of these facts, the integrated multi-trophic aquaculture (IMTA) has been proposed as a sustainable ecological approach for mitigation of the nutrient load into the aquatic environment. In such production systems, organisms from different trophic levels are co-produced and waste nutrients from fed species (e.g. fish) are assimilated and converted into valuable biomass by the extractive species (e.g. macroalgae, mussels) (Figure 1).

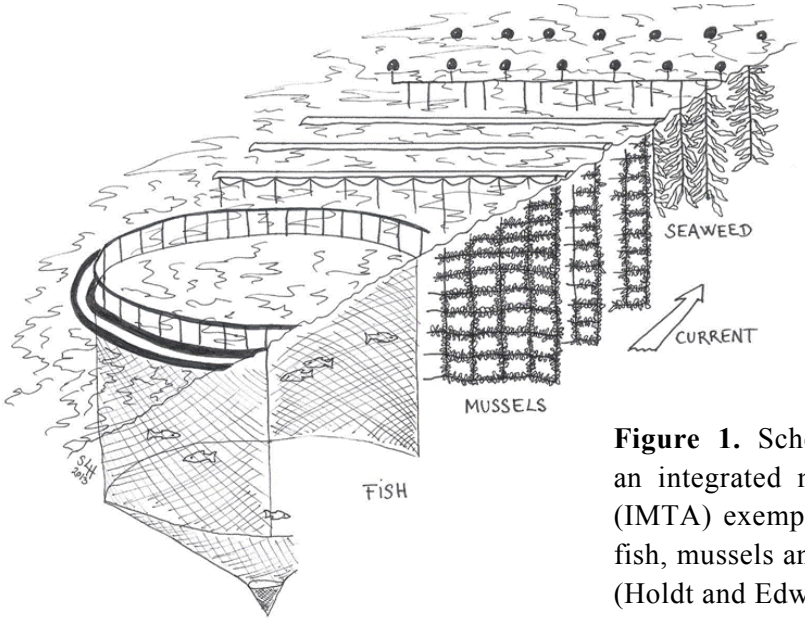


Figure 1. Schematic representation of an integrated multi-trophic aquaculture (IMTA) exemplifying the integration of fish, mussels and macroalgae production (Holdt and Edwards 2014).

Marine macroalgae, so called seaweeds, have the ability to integrate overtime changes in the environmental nutrient concentration, which makes it a suitable candidate as bioindicator of inorganic nutrients as well as biofilter for nutrient mitigation. In this context, several studies have investigated the bioremediation potential of macroalgae in both land-based and open water IMTA systems (Chopin et al. 2001; Troell et al. 2003; Neori et al. 2004; Matos et al. 2006; Abreu et al. 2011). Regarding brown macroalgae, the genus *Laminaria* sensu lato (*Laminaria* and *Saccharina*) is one of the most studied, selected based on their high bioremediation potential, yield, growth rate and commercial demand (Sanderson et al. 2012; Reid et al. 2013; Handå et al. 2013). The reported yield of brown macroalgae produced by rope cultivation from worldwide experiences ranges from 12 to 60 t DW ha^{-1} $year^{-1}$, equivalent to 80-400 t FW ha^{-1} $year^{-1}$ (Bruton et al. 2009). The highest values were found in Asia while in Europe there is lack of values for yield in up-scaled experiments. Moreover, the bioremediation capacity for *Laminaria* species based on conservative production figures found in literature is estimated at 576 kg N ha^{-1} $year^{-1}$ (Holdt and Edwards 2014).

Laminaria (kelp) biomass is primarily used as raw material for the phycocolloid industry and human consumption (McHugh 2003), however, recently there has been an increasing interest for its application as feed to other aquaculture crops (Troell et al. 2006; Nobre et al. 2010) or as substrate for the production of bio-based biofuels and chemicals (Adams et al. 2009; Vivekanand et al. 2012; Alvarado-Morales et al. 2015).

Most important macroalgae used as human food include the red macroalga *Porphyra* spp. (nori), and the brown macroalgae *Saccharina japonica* (kombu) and *Undaria pinnatifida* (wakame) with a combined aquaculture production of 9.6 million tons in Asia in 2012 (FAO 2015). *Saccharina latissima* is a close relative to the Asian kombu, which similarly is considered an edible macroalga, and has the potential to be produced through commercial aquaculture in Europe (Sanderson et al. 2012; Peteiro and Freire 2013).

The chemical composition of macroalgae varies markedly according to the species, season, geographic distribution and even population (e.g. Ito and Hori 1989; Fleurence 1999; Schiener et al. 2014). The absence of systematic data on the seasonal variations in the biomass yield and N and P contents of *S. latissima* makes it difficult to evaluate the bioremediation potential of the species year-round, upon different harvest times. Moreover, although *S.*

latissima is commercialized as human food, there is lack of data on the seasonal variations in the concentration of protein, total lipids and tract elements, and composition of amino- and fatty acids; all important parameter when evaluating the nutritional value of foods. Furthermore, the development of epiphytes, organisms that grow on the thallus of some macroalgae species, also needs to be accounted for when evaluating the value and application of the produced biomass. The commercial harvest of *S. latissima* is generally undertaken in the spring (until May) just before the epiphytic growth takes place, which is observed in late spring and summer, and makes the biomass unsuitable for human consumption. This may not be, however, the most suitable harvest time considering the bioremediation service and/or the nutritional value of the biomass. Alternatively, macroalga biomass including epiphytes may potentially be utilized as feedstock for biorefinery (e.g. feed, fuels, chemicals, products).

Macroalgae are an abundant renewable resource with a carbohydrate content that can reach up to 60% of their dry weight (DW) which make them an attractive feedstock for fermentation-based fuels and chemicals (Jung et al. 2013; Kraan 2013). Moreover, production of marcoalgae does not require agricultural land, freshwater or fertilizers and thus do not compete for resources with land-based food and feed crops. In these context, production of biofuels such as bioethanol and biogas from *S. latissima* biomass has been previously studied (Adams et al. 2009; Vivekanand et al. 2012). However, there are no studies on the potential of this species as feedstock for the production of bulk chemicals such as succinic acid. Succinic acid is a building block chemical which has attracted increasing attention due to its potential to be used as a platform for the production of a wide range of valuable commodities (Zeikus et al. 1999). Moreover, while using renewable biomasses as feedstock, fermentation-based succinic acid production also consumes CO_2 (McKinlay et al. 2007), which constitutes a remarkable feature with potential to improve the environmental and economic indicators of a biorefinery facility through process integration (Gunnarsson et al. 2014). Succinic acid production from the brown macroalga *Laminaria digitata* has been demonstrated to be feasible with high product yield and relatively high titer (Alvarado-Morales et al. 2015). Moreover, the concentration of proteins and total lipids in the solid residue recovered after the enzymatic hydrolyses were 3.5 and 8.6 times higher, compared to the original macroalga, which may increase its nutritional value. On the other hand, seasonal changes in the carbohydrates content as well as protein and total lipids of macroalgae will

have an important impact in the overall process, which needs to be accessed if a commercial process is to be considered.

The need for a systematic/comprehensive evaluation of *S. latissima* bioremediation capacity along with the valorisation of the produced biomass through the diversification of different applications motivated the investigation that lead to this PhD thesis.

1.2 Objectives, hypotheses and structure of the thesis

The main objective of this PhD research project was to optimize the bioremediation capacity of *Saccharina latissima* targeting the recovery of N and P, considering harvest time, biomass yield and N and P content. Additionally, biomass valorisation was performed through the evaluation of its chemical composition and nutritional value for especially human food and animal feed, and testing its potential as feedstock for biorefinery.The specific objectives include:

- Determine seasonal variations in the biomass yield, and N and P contents for optimization of the macroalga biofilter.
- Evaluate seasonal variations in the content of protein, total lipids and trace elements, and composition of amino acids and fatty acids.
- Trials on the protoplast cloning method for regeneration and germination of *S. latissima* cells.
- Test the potential of *S. latissima* as feedstock for fermentation-based succinic acid production in a biorefinery approach.

These specific objectives were set up, because it was hypothesized that:

- N and P content in the macroalga will change seasonally in response to variations in the environmental nutrient background concentrations.
- N and P content, and biomass yield, of macroalga cultivated close to the fish farm may increase in response to the nutrient loading from the fish farm.
- Overall chemical composition and nutritional value will change seasonally (and possibly in regard to location) in response to environmental conditions.

- The biomass including epiphytes may change the biomass composition, and the applicability, but may be utilized as feedstock for biorefinery.
- Harvest time will have an important impact on the biomass value/application (e.g. bioremediation, food, feed, biorefinery).
- Protoplast method can be a mean for regeneration of clones usable for breeding in order to optimize yield and possibly protein content of future *S. latissima* strains.

In Chapter 2, results on the commercial cultivation and bioremediation of *S. latissima* are given. The effect of cultivation site and season of harvest on growth, biomass yield, N and P contents and overall bioremediation capacity was evaluated. Most of the findings of Paper I are presented in this chapter.

In Chapter 3, the content of protein, total lipids and trace elements, and the profiles of amino acids and fatty acids of *S. latissima* were evaluated, and its potential use for human food and/or animal feed, also considering safety regulation, is discussed. The findings of Papers II and III are presented in this chapter.

In Chapter 4, the potential of *S. latissima* as feedstock for succinic acid production in a biorefinery approach was evaluated, and a novel biorefinery concept is proposed. Moreover, an overview of the prospects for macroalgae-based fuels, chemicals and products is given. Most of the findings of Paper IV are presented in this chapter.

2 Macroalgae production

2.1 World aquaculture macroalgae production

Global cultivated aquatic algae, mostly macroalgae, reached a production of 23.8 million tonnes in 2012, corresponding to a market value of US$6.4 billion (Table 1). Cultivated tonnage was almost 22 times that harvested from wild stocks (1.1 million tonnes). China, Indonesia and Philippines were the major macroalgae farming countries, with a production of 12.8, 6.5 and 1.8 million tonnes, respectively, corresponding to 89% of the global production. Other important producers include traditional macroalgae farming countries such as Republic of Korea, Korea DPRp and Japan (FAO 2014a). Most produced macroalgae include the Japanese kelp *Laminaria japonica*, Wakame *Undaria pinnatifida*, *Gracilaria* spp. and Nori nei *Porphyra* spp. cultivated mainly in China, *Eucheuma* spp. cultivated mainly in Indonesia and *Kappaphycus alvarezii* cultivated mainly in the Philippines (FAO 2015). *Laminaria japonica*, *Undaria pinnatifida*, and *Porphyra* spp. are primarily used for direct human consumption, while a significant amount of *Laminaria japonica* is also used for the extraction of alginate, mannitol and iodine (McHugh 2003; Bixler and Porse 2010). *Gracilaria* spp. is a primary feedstock for agar extraction, whereas *Eucheuma* spp. and *Kappaphycus alvarezii* are almost the exclusive sources of kappa carrageenan (Bixler and Porse 2010). Compared to the global production figures, macroalgae production in Europe is almost negligible (1.0%). Norway and France are the main macroalgae producers with a combined annual production of 181,565 tonnes, mostly provided from harvesting of wild stocks (FAO 2014a).

Table 1. World production (wet tonnes) of wild stock harvest and cultured macroalgae plus monetary values (US$) in 2012 by the 15 top macroalgae producing countries (FAO 2014a).

Harvest of wild stock			**Aquaculture**				
Source	Production (tonnes)	% of Total	Source	Production (tonnes)	% of Total	Value US$1.000s	$/ton
World Total	1 107 381	100.00	World Total	23 776 449	100.00	6 369 639	267.90
Chile	436 035	39.38	China	12 832 060	53.97	2 852 190	222.27
China	257 640	23.27	Indonesia	6 514 854	27.40	1 347 538	206.84
Norway	140 336	12.67	Philippines	1 751 071	7.36	231 735	132.34
Japan	98 514	8.90	Korea Rep	1 022 326	4.30	391 705	383.15
France	41 229	3.72	Korea DPRp	444 300	1.87	66 645	150.00
Ireland	29 500	2.66	Japan	440 754	1.85	1 277 083	2 897.50
Iceland	18 079	1.63	Malaysia	331 490	1.39	64 406	194.29
South Africa	14 509	1.31	Vietnam	234 600	0.99	117 300	500.00
Canada	13 833	1.25	Zanzibar	150 876	0.63	1 915	12.69
Korea Rep	10 123	0.91	Salomon Is	13 000	0.05	530	40.77
USA	9 382	0.85	Kiribati	8 280	0.03	600	72.46
Indonesia	7 641	0.69	Tanzania	6 510	0.03	164	25.19
Russian Fed	6 597	0.60	Denmark	5 000	0.02	2 591	518.20
Mexico	5 725	0.52	India	4 502	0.02	169	37.54
Morocco	5 150	0.47	Chile	4 126	0.02	9 512	2 305.38

2.2 Commercial cultivation and bioremediation

The reported yield of brown macroalgae produced by rope cultivation from worldwide experiences ranges from 12 to 60 t DW ha^{-1} $year^{-1}$, equivalent to 80-400 t FW ha^{-1} $year^{-1}$ (Bruton et al. 2009). The highest values were found in Asia while in Europe there is lack of values for yield in up-scaled experiments.

Macroalgae have the ability to integrate over time variations in the water nutrient concentration, which makes it a potential biofilter for inorganic nutrients. Increased nitrogen tissue content has been reported in macroalgae cultivated in the surrounding area of fish farms (Chopin et al. 2000; Abreu et al. 2009) even in situations of rapid N dilution (Buschmann et al. 2008). In this context, several studies have investigated the bioremediation potential of macroalgae in IMTA; including, red and green macroalgae, tested widely in both land-based and off-shore IMTA systems, and brown macroalgae, tested mainly in off-shore IMTA systems (Chopin et al. 2001; Troell et al. 2003;

Neori et al. 2004; Matos et al. 2006; Rodrigueza and Montaño 2007; Abreu et al. 2011). Regarding brown macroalgae, the genus *Laminaria* sensu lato (*Laminaria* and *Saccharina*) is one of the most studied, selected based on their high bioremediation potential, growth rate and commercial demand (Sanderson et al. 2012; Reid et al. 2013; Handå et al. 2013).

In paper I the bioremediation potential of *Saccharina latissima* was evaluated over a 12-month cultivation period based on the biomass yield, and P and N content and removal. *S. latissima* was cultivated at two commercial cultivation sites granted to Hjarnø Havbrug A/S outside but in vicinity of Horsens Fjord, in the inner Danish waters. The IMTA site (55° 47.529′ N, 10°03.027′ E) was located approximately 100 m away from a blue mussel farm (35 tubes including nets) and 500 m from a rainbow trout (*Oncorhynchus mykiss*) farm (175 t $year^{-1}$). While the reference site (55° 49.045′ N, 10° 06.824′ E) was located at approximately 2,000 m from the fish farm.

The maximum biomass yield (kg FW m^{-1} of cultivation lines) over one growing season was achieved in August (1.08 and 1.51) and September (0.92 and 1.49), with significantly higher values found at the IMTA site compared to the reference site in August. Similarly, the highest nutrient removal was found in August (5.07-7.02 g N m^{-1} and 0.86-1.23 g P m^{-1} of cultivation line) and September (4.73-7.24 g N m^{-1} and 0.83-0.96 g P m^{-1}); being significantly higher in the IMTA site compared to the reference site in August (Figure 2). Difference in the nutrient removal between sites was driven by the difference in the biomass yield, since the N content was similar or even higher at the reference site compared to the IMTA (July-November). The yields found in the present study are below the range of values previously reported for *S. latissima* cultivated in monoculture and in IMTA in Spain (4-16 kg FW m^{-1} of cultivation line) and Canada (11-17 kg FW m^{-1} of cultivation line), respectively (Chopin et al. 2004; Peteiro et al. 2006; Peteiro and Freire 2013). This may be explained by a combination of non-optimal environmental conditions for growth of *S. latissima* observed in the cultivation areas: water temperature was below 0 ºC during the main growth period and over 23 ºC during summer, which is outside the range of optimal growth temperature (10-15ºC) (Fortes and Lüning 1980; Bolton and Lüning 1982); salinity reached a minimum of 12-15 psu from February to April while optimal growth is found at 23-31 psu, and this species experiences pronounce growth reduction at 16 psu (Bartsch et al. 2008); and irradiance during December-February reached down to 48 µmol $m^{-2}s^{-1}$, while the photosynthetic saturation

level for kelp is around 150 µmol $m^{-2}s^{-1}$ (Lüning 1979). Furthermore, these yields are comparable to those reported for *S. latissima* cultivated in Limfjorden, Denmark (1.1-2.5 kg m^{-1}; Nielsen 2015). However, the authors reported massive crop loss due to heavy biofouling (epiphytes) during summer time, while in the present study biomass outstand epiphytic growth and cultivation was followed over a second growing season.

Macroalgae cultivation over two growing seasons enhanced the biomass yield, with the maximum values achieved in May 2014 (3.0-3.2 kg FW m^{-1} of cultivation line over 16 months). This yielded twice as much biomass compared to one single growing season; however, the biofiltration capacity was not enhanced, due to the lower N content found in May.

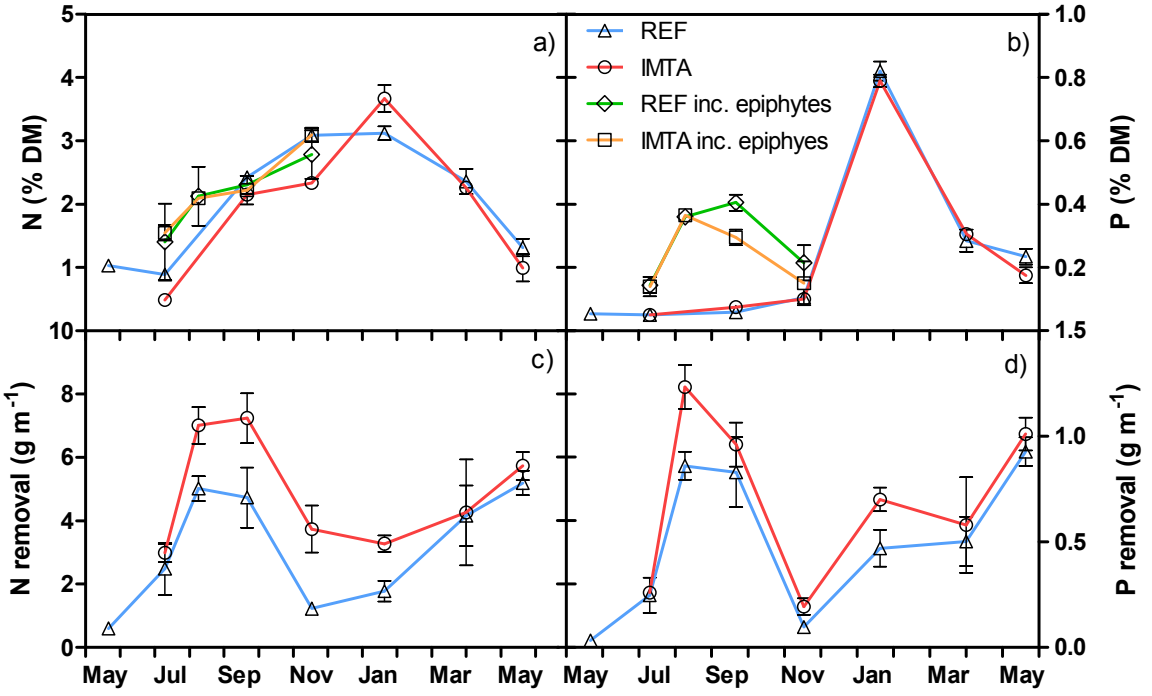


Figure 2. Year-round variation in the a) nitrogen (N) and b) phosphorous (P) content (%DM) and c) N and d) P removal (g N and P m^{-1} of cultivation line) of *Saccharina latissima* cultivated at the reference (REF) and IMTA site in 2013-2014. The N and P content of macroalga including epiphytes is also presented. Deployment at sea: 15 January 2013. Values are mean±SE (n=3).

The estimated extrapolated areal yields for *S. latissima* range from 30.4-45.6 t FW over a growing season for pilot-scale cultivation in Spain (Peteiro and Freire 2013; Peteiro et al. 2014) to 220-340 t FW ha^{-1} $year^{-1}$ for small-scale cultivation in Scotland (Sanderson et al. 2012). In this study (Paper I), a maximum extrapolated yield of 7.1 t FW ha^{-1} was achieved, corresponding to a N removal of 39.4 kg ha^{-1} over one growing season (September 2013) and 26 kg ha^{-1} over two growing seasons (May 2014) (Table 2). The bioremediation potential reported herein is below estimated values based on conservative production figures from literature (576 kg N ha^{-1} $year^{-1}$; Holdt and Edwards 2014). On the other hand, these values are higher, but comparable, to those reported in another study in Danish waters (15.4 kg N ha^{-1} $year^{-1}$; Nielsen 2015), suggesting a lower bioremediation potential of *S. latissima* in Denmark, compared to other European countries. Based on the tonnage of the specific fish farm (175 t $year^{-1}$), assuming a feed conversion ratio of 1.2:1 and that 3.83% of feed ends up as dissolved nitrogen (Black 2001) we estimate that 0.29-0.49% of the feed-N could be recover by 1-ha macroalgae biofilter harvested in August and/or September and that a 204-340-ha macroalgae cultivation area would be needed to achieve 100% N recovery. These results highlight the discrepancy between area required for fish and macroalgae biofilter production. The suggested extrapolated yields and bioremediation capacity in this study were based on the results from large-scale cultivation which should provide realistic values for *S. latissima* commercial cultivation, especially in North European environmental conditions. Moreover, the features of the cultivation system will have an important impact on the estimated and actual yields and it will influence the meter of cultivation line per ha. In this study cultivation of *S. latissima* was done at up to 5-6 m depth without significant effect on growth (data not shown), while in Linfjorden hampered growth was observed at depth deeper than 2 m, due to high chlorophyll a concentrations and turbidity associated with highly eutrophic waters (Nielsen 2015). Hjarnø Havbrug A/S is now testing the potential to increase the areal yield of *S. latissima* by cultivating the macroalga at higher depths (>6m; personal communication, Teis Boderskov).

Table 2. Biomass yield (kg FW m^{-1} of cultivation line), estimated areal yield (tonnes FW ha^{-1}) and N removal (kg ha^{-1}) of *Saccharina latissima* cultivated at both reference (REF) and IMTA site upon different harvest times.

	Harvest time	Cultivation site	
		IMTA	REF
Harvested biomass (kg FW m^{-1} line)	May13		0.41
	Aug13	1.02	0.99
	Sep13	1.30	1.01
	May14	1.23	1.42
Harvested biomass (t FW ha^{-1})	May13	-	2.1
	Aug13	5.5	5.0
	Sep13	7.1	5.1
	May14	6.7	7.1
N removal (kg ha^{-1})	May13	-	3.0
	Aug13	38.2	25.1
	Sep13	39.4	23.7
	May14	31.2	26.0

All cultivation lines were from the same nursery/reproduction batch and were deployed at sea in January 2013. Data were computed using data on biomass yield measured from 4 m in length cultivation lines (droppers), the N content of the biomass collected in the first meter of line (1-2 m depth) and meters of cultivation line per ha based on current features of the cultivation system.

The maximum and minimum N contents found in the present study followed the maximum and minimum ambient nitrate concentrations. This profile was also found for P. Moreover, the N content of the macroalgae cultivated at the reference site was higher than that of those cultivated at the IMTA site (Figure 2). This data suggest that the inorganic nitrogen released from the fish farm is negligible compared to the naturally occurring background concentration of nitrate. This may be explained by the characteristics of the specific IMTA system: the fish farm tonnage is relatively small compared to other commercial salmonid farms; and the macroalgae cultivation site is 500 m away from the fish cages and located downstream only 50% of the time.

In this study growth of epiphytic organisms started in July, reached its maximum in September and remained until November. The main epiphytes included bryozoans but also barnacles, filamentous macroalgae, and blue mussel juveniles. The presence of epiphytes makes the biomass unsuitable for human consumption (Figure 3). However, it may have potential to be used as

an ingredient for animal feed (Paper II and III), or as feedstock for biorefinery (Paper IV) which requires further investigation.

Figure 3. *Saccharina latissima* harvested in a) May representing clean macroalgae and b) September representing macroalgae covered by epiphytes (Photos: Cátia Ribeiro).

This study gives comprehensive information of the bioremediation potential of *S. latissima* cultivated commercially in the inner Danish waters. Harvest in August-September in an annual cultivation will result in maximum nutrient removal. On the other hand, highest epiphyte-free biomass will be found in May. Biannual cultivation (two growing seasons) will increase biomass yield but without enhancing the nutrient removal. This indicates an apparent mismatch between maximum biofiltration capacity and biomass value for human consumption. Selection of harvest time will depend on the bioremediation efficiency, biomass yield and epiphytic coverage which have an important impact on the application/value of the produced biomass.

2.2.1 Future perspectives for macroalgae cultivation in Europe

Cultivation systems for *Laminaria* species in Europe have mainly been using farming technology imported from Asia (e.g. rope cultivation, longlines). Such cultivation technics are labour intensive and time consuming. Research and development projects on macroalgae cultivation in Europe are now focused on the development of innovative cultivation structures that promise to increase considerably macroalgae yields. This is the case of the project AT~SEA and the company Energy Seaweed Solutions which have been developing 3D textile substrates for macroalgae cultivation (SEA~AT 2015;

SES 2015). The development of cost-effective methodologies for deployment at sea and harvest of such structures will be of primordial importance for the establishment of such cultivation systems in Europe; which will largely require mechanization. Moreover, direct seeding just before deployment at sea is also being considered (Mols-Mortensen 2015; SEA~AT 2015), this would allow skipping the land-based hatchery step, and thus reducing considerably the production cost of macroalgae. However, the challenge lies on preventing the spores/gametophyes/sporophytes from readily being washed away at deployment by the currents, and outgrown by epiphytic organisms when set in sea at such an early development stage.

Farmed macroalgae production almost doubled between 2000 and 2012 in China, which is partially due to the development of high-yield strains of major species (FAO 2014b). Intraspecific and interspecific cross-breeding of gametophyte clones has been used as a tool to produce high yielding and high temperature-resistant sporophytes, which is the environmentally limiting factor for seasonal restriction of *Laminaria* production in China (Zhang et al. 2007; Li et al. 2008; Zhang et al. 2008).

Similarly, the establishment of breeding programs for macroalgae domestication towards the development of improved commercial strains (e.g. palatability, higher yield, higher tolerance to temperature and salinity fluctuations, and less prone to epiphytic growth), may in the future contribute significantly for the optimization of macroalgae production and bioremediation capacity in Europe. Moreover, it could also reduce the size/weight heterogeneity currently observed for individuals produced using spores from wild reproducers (Paper I).

In this project, the potential to isolate and regenerate protoplasts from *S. latissima* to be used as a breeding technique was briefly investigated. These technique has been successfully tested in a number of low differentiated macroalgae in which the isolated protoplasts were able to regenerate the cell wall and regenerate into a new individual (Reddy et al. 2007). However, protoplast regeneration from highly differentiated macroalgae (e.g. *Laminaria*) has a lower success rate (Mussio and Rusig 2009). In the present study, protoplasts were successfully isolated from *S. latissima* somatic tissue after enzymatic hydrolysis with a combination of cellulose and an alginate lyase (Aly A1), or cellulase and two alginate lyases (Aly A1 and Lyase pseudomonas), with no different between treatments. Protoplast recovery after hydrolysis was performed at different centrifugation

speeds (50-300G) and times (5-12 minutes). A maximum protoplast yield of $77.8x10^6$ protoplasts per g of macroalga was achieved. The isolated protoplasts recovered after centrifugation at 50G for 12 minutes, survived for 1 week; however, after that mass mortality was observed (Figure 4). Higher centrifugation speeds would further reduce the survival time of the protoplasts. In these trials protoplast regeneration was not observed. These results from preliminary trials confirm the limitations of protoplast-to-plant regeneration from *S. latissima* sporophytes (Benet et al. 1997).

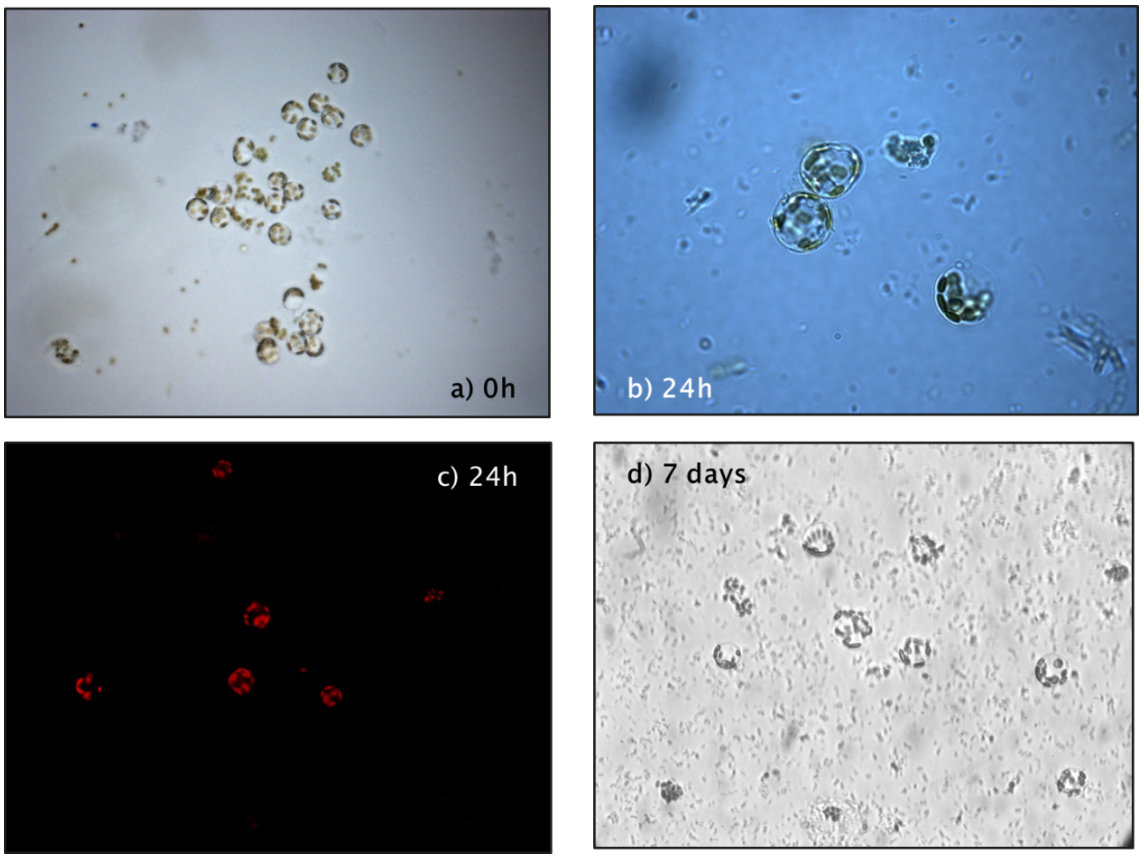


Figure 4. Protoplasts right after isolation, and 24 hours and 7 days after isolation; under bright field (a, b and d) and green florescent light (c) (Photos: Gonçalo Marinho).

3 Biomass chemical composition and nutritional value

Macroalgae are a valuable source of vitamins, minerals, lipids, protein, and dietary fibres (Holdt and Kraan 2011; Tabarsa et al. 2012). Moreover, macroalgae have low caloric value, which makes them suitable as dietetic food, and contain high amount of dietary fibres, whose consumption is known to reduce the occurrence of chronic diseases such as diabetes, obesity, heart diseases and cancer (Southgate 1990).

Macroalgae have been used for both human and animal consumption since ancient times, especially in China, Japan and Republic of Korea (Černá 2011; Evans and Critchley 2013). On the other hand, in Europe macroalgae have been and are still mainly used for the extraction of phycocolloids, thickening and gelling agents used in a wide range of industrial applications, including food (Mabeau and Fleurence 1993; McHugh 2003). However, macroalgae were recently introduced in the cuisine as a healthy food of several American and European countries (McHugh 2003). In France the use of macroalgae as sea vegetables and condiments for food has been regulated few decades ago (Mabeau 1989). Whilst in other European countries there is no specific legislation for the utilization of macroalgae species for human consumption, specific species are authorized if commercialized as food or food ingredient and consumed to a significant degree before May 15 1997 (Holdt and Kraan 2011). Nevertheless, in western countries, the utilization of macroalgae as high nutritional value food is still very little explored.

In this chapter chemical and nutritional characterization of *S. latissima* biomass (protein, amino acids, lipids, fatty acids, carbohydrates and minerals) was performed and its application for especially human food and animal feed is discussed.

3.1 Protein and amino acids

Growth of human population has driven the need for alternative food sources (Rosegrant and Cline 2003). Protein-energy malnutrition is a main health problem reaching both children and adults, especially in developing countries (Stephenson et al. 2000). The global production of marine macroalgae reached 24.9 million tonnes in 2012 (FAO 2014b). The protein content of macroalgae ranges from 3-21%, 10-26%, and 8-47% of dry weight in brown, green and red macroalgae species, respectively (Fleurence 1999; Holdt and

Kraan 2011). Moreover, macroalgae protein contains all amino acids especially glycine, alanine, arginine, proline, glutamic and aspartic acids. Glutamic and aspartic acids constitute a large amount of total amino acids ranging from 15 to 44% (Černá 2011). On the other hand, threonine, lysine, tryptophan, sulphur amino acids (cysteine and methionine) and histidine are usually the limiting amino acids, even though their levels are generally higher than those found in vegetables and cereals (Holdt and Kraan 2011). Amino acids are usually classified as: essential amino acids (EAAs), which cannot be synthesized by the organisms and therefore must be obtained from the diet; or non-essential amino acids, which can be synthesized by the organisms, and thus are considered not required in their diets. EAAs in human nutrition include leucine, isoleucine, valine, lysine, threonine, tryptophan, methionine, phenylalanine and histidine (Friedman 1996; WHO/FAO/UNU 2007), while arginine is also an EAA in diets for aquaculture crops such as fish and abalone (Metailler et al. 1973; Allen and Kilgore 1975; Hardy 2002). In some macroalgae species essential amino acids can account for almost half of the total amino acids (Černá 2011) and the amino acid score of edible macroalgae such us *Saccharina latissima* (82), *Porphyra complex* (Amanori) (91) and *Undaria pinnatifida* (Wakame) (100) is comparable to that of beef (100) (Murata and Nakazoe 2001).

Fishmeal has long been used as the main source of protein in aquafeeds; however, this resource is already showing signs of overexploitation (FAO 2012). In this context, the replacement of fishmeal by vegetable protein sources, such us soybean, corn gluten, cotton seed, and canola has been extensively studied (see review by El-Sayed and Tacon 1997). Comparatively, macroalgae have been given less attention, even when they make part of the food chain of some fish species and their production does not require agricultural land, fertilizers and freshwater supply (Marinho et al. 2013). Dietary deficiencies or imbalanced amino acid profiles have been pointed out as one of the most important factors leading to pour fish growth performance when fish meal is completely or partially replaced for alternative vegetable protein sources (Dias et al. 2005; Goda et al. 2007; Silva et al. 2010). The amino acid score of macroalgae ranges from 60-100%, which is higher than that of common vegetable protein sources (Murata and Nakazoe 2001; Černá 2011; Holdt and Kraan 2011). Nevertheless, Azaza et al. (2008) found that Nile tilapia (*Oreochromis niloticus*) diet containing 30% of *Ulva* meal (*Ulva rigida*) was deficient in phenylalanine, methionine, cysteine and threonine according to the species' requirements, relating that

with the impaired growth performance. Moreover, most studies concluded that the inclusion level of macroalgae meal in fish diets without depression of growth performance should be limited to 10% (Valente et al. 2006; Soler-Vila et al. 2009; Marinho et al. 2013).

Chemical composition for nutritional value of macroalgae, including protein content, varies markedly according to the species, season, geographic distribution and even population (e.g. Ito and Hori 1989; Fleurence et al. 1999). Protein content of *S. latissima* is higher in February-March (13-14% dry weight), while lowest values are found during July-September (5-8% dry weight) (Black 1950). Although the amino acid composition of *S. latissima* has been reported before (Mai et al. 1994; Murata and Nakazoe 2001) no information is available on the seasonal changes in the amino acid composition of this species.

In paper II the effect of harvest time and cultivation site on the protein content and nutritional value, based on the amino acid composition, was evaluated. The protein concentration varied significantly from a minimum of 1.43% DW in May 2013 to a maximum of 10.8% DW in November (Figure 5).

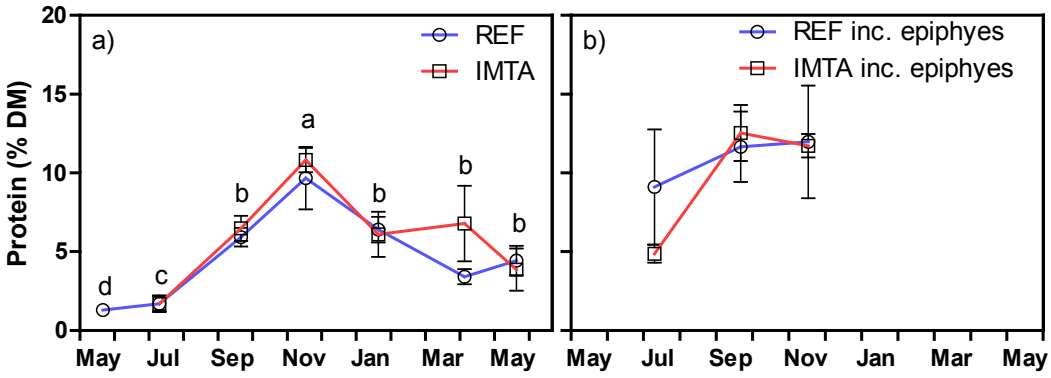


Figure 5. a) Year-round variation in the protein content (%DM) of *Sacharina latissima* cultivated at the reference (REF) and IMTA site; b) also including epiphytes when present (July-November). Values are mean±SE (n=3). Different letters identify significant differences between sampling months (p<0.05).

Our results confirmed macroalgae as a source of "all amino acids". With exemption of tryptophan, destroyed due to the acid hydrolysis, all of the other 18 analysed amino acids were found in most of the samples, regardless the cultivation site or harvest time. Aspartic and glutamic acids together accounted for 19-26% of total amino acids in July and 42-49% in March

(p<0.05). There were significant seasonal changes in the amino acid composition of *S. latissima*, while cultivation site did not show a significant effect. Greatest seasonal differences in amino acid composition occurred in July, with leucine contributing most (22.7–26.7 %) of the observed differences.

Highest protein content reported for *S. latissima* in this study is comparable to the protein content found in wheat meal; moreover, its protein presented higher concentrations of lysine, methionine and arginine than that of wheat meal suggesting that the replacement of wheat meal by *S. latissima* meal could be possible without impairing dietary protein content or amino acid composition. On the other hand, compared to standard protein ingredients for fish feed such as fish meal and soy meal *S. latissima* biomass presents lower protein content and the concentration of highly desirable amino acids such as lysine and methionine are lower. Biomass including epiphytes (July-November) presented a similar amino acid content and EAA score, but reduced EAA content and EAA ratio compared to clean macroalgae biomass. The nutritional value of proteins referred to as amino acid score was evaluated based on the composition of EAA. Histidine was the first limiting EAA year-round with exception of the samples collected in May 2013. Biomass harvested in November achieved the maximum EAA score (68.9%, based on requirement pattern of WHO/FAO/UNU (2007)); which is within the range of values reported for edible macroalgae in Japan (60-100%; Murata and Nakazoe 2001) and higher than that of oats, rice, soybeans, wheat or peanuts (43-57%; Brody 1999).

Considering that higher yield and clean biomass were found in May (Paper I) and that maximum protein content and EAA score were found in November, there is an apparent mismatch between harvest time for maximum yield of biomass suitable for human consumption and nutritional value.

3.2 Total lipids and fatty acids

Vertebrates are unable to synthesize linoleic (18:2n-6) and α-linolenic acid (18:3n-3) (van Ginneken et al. 2011). Furthermore, conversion of the precursor α-linolenic acid to the physiological essential long-chain polyunsaturated fatty acids (LC-PUFA) such as EPA and DHA is restricted in humans (Gerster 1998) and fish, particularly in marine species (Owen et al. 1975; March 1993). Appropriate consumption of LC-PUFA has been shown to reduce the risk of cardiovascular diseases, cancer, depression and mental illness, while promoting the normal development of the nervous system

(Simopoulos 2002; Simopoulos 2008). Over-pressure on fish stocks, the traditional source of n-3 LC-PUFA, makes it necessary to explore the potential of alternative foods for the provision of these PUFA. Although macroalgae present generally low concentrations of total lipids (0.61-6.48%; Fleurence et al. 1994), they contain high proportions of polyunsaturated fatty acids (PUFA). Both red and brown macroalgae contain LC-PUFA's arachidonic acid (ARA; 20:4n-6) and EPA as main PUFA's. On the other hand, these PUFA are present in lower levels in green macroalgae as well as terrestrial plants (Fleurence et al. 1994; van Ginneken et al. 2011; Schmid et al. 2014).

In paper III the effect of harvest time and cultivation site on the total lipid content and fatty acid composition was evaluated. The total lipid content varied seasonally from 0.62-0.88% DW in July to 3.33-3.35% DW in November, while no significant difference was found between cultivation sites. Similarly, the fatty acid composition was not affected by the cultivation site while seasonal changes were observed. The fatty acid composition in January differed most from all the other sampling months, where changes in the relative abundance of 20:5n-3, 14:0 and 18:1n-9 explained most of the dissimilarities; 13.12%–33.35%, 11.07%–29.37% and 10.15%–16.94%, respectively. PUFA constituted a minimum of 31.3-33.6% FAME in January and made up to more than half of the FAME in July (53.3-54%) (Figure 6); including, the most appreciated health beneficial EPA and DHA, but also ARA and SDA. *Saccharina latissima* constitutes a better source of LC-PUFA than traditional vegetables, which generally do not contain such fatty acids. Additionally, the macroalga's FAME contains higher proportions of ARA, SDA and EPA, but lower DHA compared to fat fish (salmon), and higher proportions of ARA and SDA, but lower EPA and DHA compared to lean fish (cod).

The macroalga presented nutritionally beneficial PUFA/SFA and n-6/n-3 ratios. These data suggest that consumption of *S. latissima* may contribute to a more balance dietary PUFA/SFA and n-6/n-3 ratios. However, the low lipid content of this species resulted in low fatty acid concentrations. An estimated consumption of 1,106–1,627 g of fresh weight (166–244 g DW) would be necessary to fulfil the recommended daily intake of 250 mg of EPA/DHA (Lagiou et al. 2009). Considering the high quality, but low quantity of *S. latissima*'s FAME, fatty acid extraction should be considered if this species is to be used as a source of LC-PUFA.

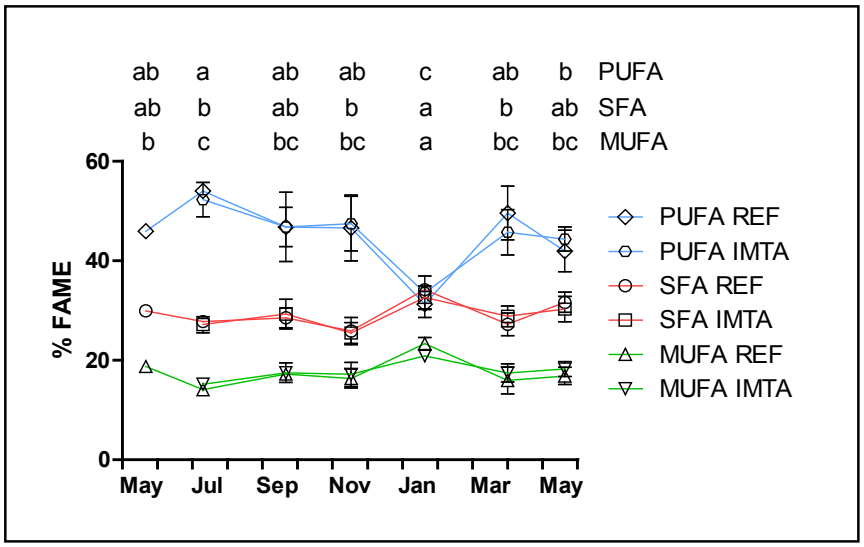


Figure 6. Year-round variation of polyunsaturated (PUFA), saturated (SFA) and monounsaturated fatty acid (MUFA) (% fatty acid methyl esters; FAME) of *Saccharina latissima* cultivated at the reference (REF) and IMTA site in 2013–2014. Biomass cleaned of epiphytes when present (July–November). Values are mean±SE (n = 3), and different letters in the same row indicate significant differences between sampling months (p<0.05).

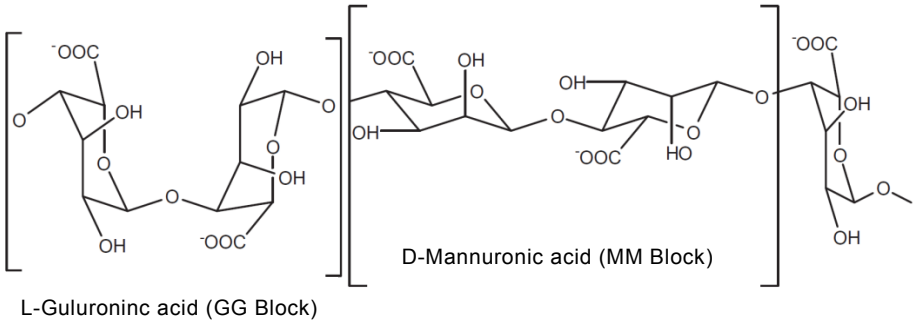
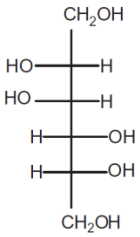
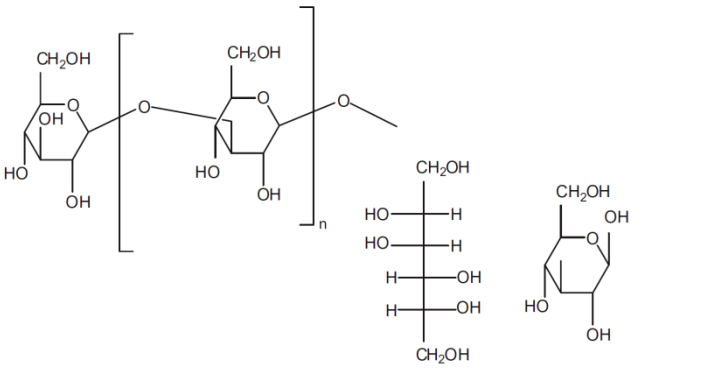
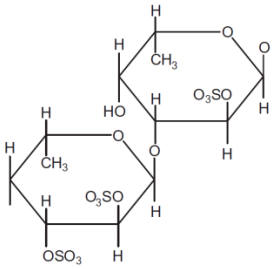
Seasonal growth of epiphytes generally increased the proportions of SFA and DHA, and reduced the n-3. *S latissima* (including epiphytes) presents lower proportion of EPA (except in November) and DHA then fish meal, while the former does not contain ARA and SDA. Considering the low concentration of fatty acids of *S. latissima* and that macroalgae inclusion in fish feed is generally limited to 10% this species may not be considered as an alternative source of fatty acids to fish meal. Regarding harvest time, January resulted in lower proportions of PUFA, n-3 and n-6 and thus must be avoided if high PUFA proportion is desired. On the other hand, biomass harvested in November and March should present the highest concentrations of n-3 as it contained high proportions of these fatty acids along with high total lipids concentration.

3.3 Carbohydrates

Macroalgae present high content of carbohydrates, however their composition vary markedly according to species and seasonal changes in the environmental conditions (Adams et al. 2011; Schiener et al. 2014). Thus characterization of carbohydrate composition is of primordial importance for the selection of suitable macroalgae feedstock for biorefinery. The carbohydrate content of green, red and brown macroalgae generally ranges from 25-50%, 30-60%, and 30-50%, respectively (Holdt and Kraan 2011;

Jung et al. 2013). Major carbohydrates found in the brown algae include alginate, laminarin, mannitol and fucoidan (Song et al. 2015). Alginate is a major component of the cell wall (structural carbohydrate) in brown algae accounting up to 40-47% of the macroalgae dry weight. Alginate is a water soluble polysaccharide consisting of a linear unbranched chain of β(1→4)-linked-D-mannuronate and α(1→4)-linked-L-guluronate residues of widely varying composition and no regular repeating unit (Table 3) (Anastasakis et al. 2011). Laminarin and mannitol are reserve carbohydrates and most readily available carbohydrates in brown algae. Laminarin is composed of 20-25 glucose unites linked together by β(1→3) bonds with β(1→6) branching. Mannitol is a more reduced alcohol form of the sugar mannose present in the monomeric form and easily extractable (Horn et al. 2000b).

Table 3. Chemical structure of major carbohydrates presented in brown algae (Anastasakis et al. 2011).

Carbohydrate	Chemical structure
Alginic acid	
Mannitol	
Laminarin	
Fucoidan	

Our results (Paper IV) showed marked seasonal variation in the concentration of glucose and mannitol of *S. latissima*, with the higher values found in spring and summer (Figure 7). This is likely related with nitrogen depletion due to the proliferation of microalgae blooms (high chlorophyll a; data not shown) and high incident light during spring and summer (Paper I), which leads to growth limitation, high photosynthetic rate and accumulation of reserve carbohydrate (i.e. laminarin and mannitol). Moreover, *S. latissima* cultivated over two growing seasons (deployed in 2012) presented significantly higher concentrations of glucose and mannitol (up to 53.6% DM combined) than the macroalga cultivated over one single growing season (up to 33.5% DM combined; deployed in January 2013) (data not shown) and harvested at the same time (July and August 2013). These results showed that not only harvest time (season), but also growth period (age) will have a dramatic effect on the availability of fermentable sugars in the macroalgae feedstock.

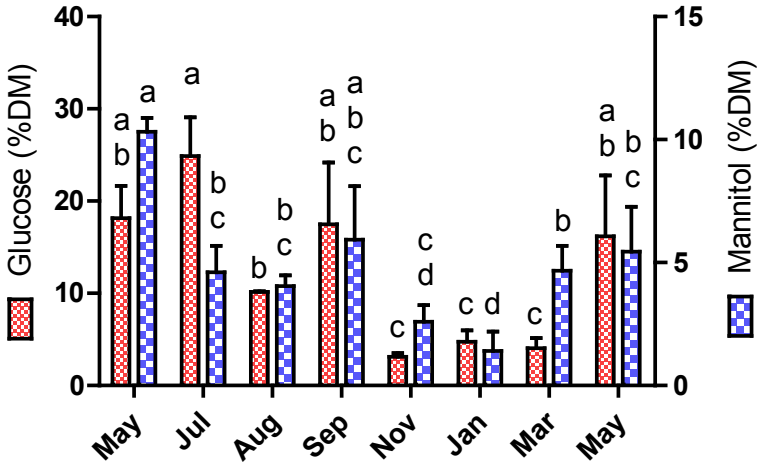


Figure 7. Year-round variation in the glucose and mannitol content (%DM) of *Saccharina latissima*. Different letters represent significant difference (p<0.05) between sampling months.

3.4 Minerals and heavy metals

Minerals are inorganic nutrients present in all body tissues and fluids and essential for live. Mineral function in vertebrates include: construction and maintenance of bone, regulation of nerve and muscle function, maintenance of osmotic pressure and pH. Moreover, they constitute essential components of enzymes, hormones, respiratory pigments, etc. (Soetan et al. 2010). Macroalgae are recognized for their ability to assimilate minerals and trace elements from the surround environment, which makes them suitable

organisms for trace element biomonitoring. The ash content of macroalgae can account up to 55% of its dry weight, this including essential macro- and microminerals. In this context macroalgae have been studied as a mineral source for animal feed (Singh et al. 2014; Rey-Crespo et al. 2014). *Saccharina latissima* ash content varied seasonally from 15.3% DM in July to 40.2% DM in January (Paper II). Na, K, Ca, Mg, P and I were present at higher concentration, followed by Fe, Mn and Zn, while Cu, Cr, Co, Se, Cd, Hg, Pb were generally found in trace amounts (<10 mg kg^{-1}).

Iodine is essential for the synthesis of thyroid hormones, which play an important regulatory function in the metabolism of humans and fish. Deficient iodine consumption is associated with disorder such as goitre and cretinism, and constitutes a global healthy problem (Pearce et al. 2013). On the other hand, iodine can also be toxic if consumed above certain levels, and thus its consumption should not exceed 600 µg d^{-1} (Nordic Council of Ministers 2008). The iodine content of several macroalgae species commonly found in the Norwegian coast ranged from 21 to 3,500 mg kg^{-1} DM, where *Laminaria* species displayed the highest values (Maehre et al. 2014). In the present study iodine content of *S. latissima* ranged from 1,194 mg kg^{-1} DM in May 2014 to 5,001 mg kg^{-1} DM in May 2013. These concentrations are much higher than the recommended value for edible macroalgae in France, while the maximum value is equal to the limit set for USA (Table 4). This result confirms kelp as an exceptionally rich source of iodine, which may be of relevance especially in areas where iodine deficiency is still a healthy problem. Moreover, dietary supplementation with macroalgae can be used to increase iodine content and thus nutritional value of fish flesh (Valente et al. 2015), another main natural source of iodine.

Our results further showed that an estimated consumption of 5g FW of *S. latissima* (0.75g DW, assuming a ratio of DW/FW of 0.15) d^{-1} would not result in the provision of significant levels of essential minerals based on their recommended daily intake (P, Zn<1.0%; Na, K, Se, Cu<3.0%), with exception for Ca (1.70-14.90%), Fe (0.89-41.05%), and I (above the upper intake value).

Besides essential macro- and microminerals macroalgae also assimilate heavy metals such as inorganic arsenic, lead, cadmium and mercury, undesirable toxic compounds that constitute a human health safety issue. Our results showed that for *S. latissima* harvested year-round the concentrations of inorganic arsenic (0.09-1.13 mg kg^{-1} DW), lead (0.4-2.81 mg kg^{-1} DW) and

mercury (0.01-0.05mg kg^{-1} DW) were below the limits for food and dietary supplements established in France, USA and EU. Cadmium concentration (0.24-0.64mg kg^{-1} DW) in August samples was above the limit established in France, but below the EU regulation. Nevertheless, in order to reach the provisional tolerable weekly intake limit set for cadmium (7 µg kg^{-1} body weight per week) (JECFA 2004) a person weighing 70 kg would need an intake of 766-2,042 g DW (equivalent to 5,104-13,611 g FW) of *S. latissima*, depending on harvest time. Regarding EU regulations for feed ingredients, concentrations of lead and cadmium were below the threshold limit values, 10 mg kg^{-1} and 1 mg kg^{-1}, respectively. However, the concentration of total arsenic for the biomass harvested from September to March (41.5-63.3 mg kg^{-1}) was above the regulatory level (40 mg kg^{-1}). That has also been reported for *Laminaria* species cultivated in Limfjorden, Denmark (Nielsen 2015). Although most of arsenic found in *Laminaria* species is in the organic and less toxic form (Raab et al. 2005), with inorganic arsenic accounting generally for less than 2% (and up to 8% for biomass including epiphytes) of total arsenic (this study), this regulation restricts the used of the biomass produced during that period as feed ingredient.

Table 4. Quality criteria applied to edible macroalgae sold in France, regulations in the USA and for dietary supplements in EU (Holdt and Kraan 2011).

	Limit (mg kg^{-1} DM, ppm)		
Toxic minerals	France	EU	USA
Inorganic arsenic	<3.0	No regulation	<3.0
Lead	<5.0	<3.0	<10.0
Cadmium	<0.5	<3.0	
Mercure	<0.1	<0.1	
Iodine	<0.5		<5,000

Overall, the daily intake of *S. latissima* is limited by the iodine content and should not exceed 0.80-3.35 g of fresh biomass, equivalent to 0.12-0.50 g DW, upon different harvest times. However, boiling kombu (*Laminaria* spp.) in water for 15 minutes can reduce its iodine content up the 99%. Indeed, processed kelp is commonly boiled in dye for 30 minutes ("ao-kombu" or "kizami-kombu"), which can reduce its iodine content prior to consumption (Zava and Zava 2011). On the other hand, when *Laminaria* is used to flavour soup stocks it results in a soup stock high in iodine (0.66-31mg L^{-1}). Thus different processing methods including cooking will impact the concentration of iodine in the final product, which requires further investigation.

4 Macroalgae-based biorefinery

Environmental concerns and safety of energy supply have been driven the quest for a shift from a fossil-based economy to a bio-based economy. In this context, biorefinery has been spotlighted as having the potential to partially or totally displace oil refinery through the production of equivalent bio-based fuels and chemicals. Biorefinery has been defined as the facility or a cluster of facilities that integrate biomass conversion processes and technologies to produce a palette of marketable products (food, feed, chemicals, and materials) and energy (biofuels, power and/or heat) from biomass in a sustainable and efficient way (Alvarado-Morales et al. 2009).

Biorefinery has been mainly utilizing energy crops such as corn and sugar cane, which increases the pressure over the global food stocks (Headey and Fan 2008). Moreover, sustainability of first generation biofuels has been questioned when considering land usage change and water consumption (Fargione et al. 2008; Dominguez-Faus et al. 2009). In this context macroalgae, have been receiving increasing attention as a more sustainable feedstock for biorefinery. Unlike land-based crops macroalgae cultivation does not require agricultural land, freshwater or fertilizers, and thus do not compete for resources with terrestrial food crops.

As mentioned macroalgae are an abundant renewable resource with a carbohydrate content that may account up to 60% of their dry weight (Kraan 2013), along with a number of bioactive compounds such as vitamins, minerals, pigments, proteins, lipids and phenols (Holdt and Kraan 2011), which makes them a very attractive feedstock for the production biofuels and biochemicals in a biorefinery approach. Moreover, due to the fact that macroalgae do not contain lignin, or only to a negligible extent, harsh pretreatment of biomass prior to saccharification may be unnecessary (Paper IV; Adams et al. 2009; Alvarado-Morales et al. 2015), and formation of lignin-originated inhibiting compounds is avoided, which presents obvious environmental, technical and economic benefits.

Although macroalgae have high potential as feedstock for biorefinery the dramatic changes in their chemical composition (e.g. seasonally; Paper I, II, III) constitute a drawback compared with terrestrial biomasses and needs to be evaluated if macroalgae are to be effectively used as biorefinery feedstock.

If macroalgae refinery aims to contribute significantly for a bio-based economy it should include the production of low price high volume products

such as biofuels and building blocks (precursors of bulk chemicals). Moreover, to improve the cost-effectiveness of the biorefinery facility it should include the production/extraction of low volume high value products such as pigments, polyphenols, polysaccharides, etc. (Figure 8).

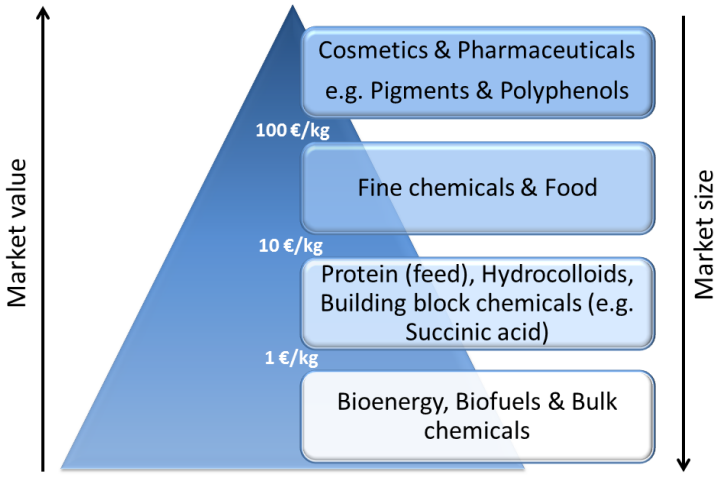


Figure 8. Value pyramid for macroalgae product markets.

4.1 Biofuels

Due to the low lipid content of macroalgae they are not considered as feedstock for biodiesel production. Moreover, since the moisture content of macroalgae is around 80-85% of its fresh weight, combustion processes result in a negative lower heating value (Bruton et al. 2009). Additional, their ash chemistry has also been reported to restrict their utilization for combustion and gasification (Ross et al. 2008). Thus macroalgae are more suitable for bio-conversion processes than for direct combustion or thermochemical conversion processes. Anaerobic digestion is considered to be the bioconversion process using macroalgae to produce energy closest to commercialization (Bruton et al. 2009). Several studies have proven the technical feasibility of anaerobic digestion of macroalgae biomass with maximum reported yields of 204-480 Nml CH_4 g^{-1} volatile solids (Song et al. 2015). Moreover, co-digestion with other organic wastes can improve the methane yield and production rate (Vivekanand et al. 2012; Song et al. 2015). Thus macroalgae can be co-digested according to seasonal availability with other common organic wastes using existing biogas plants. Although technically viable utilization of macroalgae for biogas production still requires a substantial reduction in the feedstock price (at least 75%) to

become economically viable (Bruton et al. 2009). On the other hand, production of biogas from macroalgae processing residual solids and streams, after extraction of valuable products and/or production of other fermentation products could increase the commodity portfolio and improve the economics. Anaerobic digestion of the solid residue recovered after enzymatic hydrolysis and fermentation broth from succinic acid production allowed for an energy recovery of 298 and 285 Nml CH_4 g^{-1} volatile solid, respectively (Alvarado-Morales et al. 2015).

Ethanol production from macroalgae has been extensively studied. Maximum ethanol yields from fermentation of mannitol and glucan (laminarin and trace cellulose) ranged from 0.152 to 0.196 kg (kg brown algae)$^{-1}$. However, full potential ethanol production is limited by the unique carbohydrates found in brown algae. Commercial yeast *Saccharomyces cerevisiae* is capable of fermenting glucose (laminarin monomer), but not mannitol or alginate. In this context, Horn et al. (2000) have investigated the capability of a number of microorganisms (bacteria and yeast) to ferment laminarin and mannitol; the yeast *Pichia angophorae* was the only tested organism capable of utilizing both substrates to produce ethanol. Recently, breakthrough studies on the development of genetically engineered platforms have opened the gate for full potential brown algae utilization for ethanol production. Genetic engineering tools were used by Wargacki et al. (2012) to develop a microbial platform able to co-ferment alginate, mannitol and glucan from *S. japonica* into ethanol achieving a yield of 0.281 g ethanol g^{-1} dry macroalga, and titer of 4.7% (v v^{-1}). Similarly, Enquist-Newman et al. (2014) have genetically engineered a *S. cerevisiae* platform capable of fermenting mannitol and alginate monomer to produce ethanol, with a reported titer of 4.6% (v v^{-1}). The ethanol titers achieved in these studies are comparable to the benchmark values for commercial cellulosic ethanol industries (Lau and Dale 2009). These results forecast metabolic engineering as a primer tool for the development of commercial macroalgae-based fermentation processes.

Butanol is another biofuel that can be produced from macroalgal carbohydrates *via* the so called ABE fermentation process using *Clostridium* sp. as fermentative microorganisms. Fermentation products consist of a mixture of acetone, butanol, ethanol and organic acids. Acetone-butanol fermentation by *Clostridium acetobutylicum* (ATCC 824) using glucose, mannitol and extracts of *Saccharina* spp. as carbon sources reached butanol and total solvent yields of 12% and 16% (g g^{-1} of total sugars), respectively (Huesemann et al. 2012). These results showed that significant optimization

to this process is required if macroalgae biomass is to be considered as potential feedstock for ABE production.

4.2 Biochemicals and bioproducts

Human food constitutes the main market for macroalgae, with an annual global value of $5 billion. Hydrocolloids such as agar, carrageenan and alginate are the main commercial products extracted from red and brown macroalgae, used as thickening and gelling agents. Hydrocolloid extraction industry dates back to the 1930's and constitute presently a global market value of $585 million annually (McHugh 2003). Other relatively minor commercial applications for macroalgae feedstock include fertilizers and soil conditioners, and animal feed both with a market value of $5 million on an annual basis (McHugh 2003).

Brown macroalgae particularly *Laminaria* species constitute a rich source of iodine (Maehre et al. 2014). The iodine content of *S. latissima* measured in the present study ranged between 1,194-5,001mg kg^{-1} DM. In the mid-19 century there was an established industry producing iodine from macroalgae in Glasgow (Scotland), which was later overpassed by cheaper alternatives. Currently, it is estimated that less than 20% of Japanese kelp *L. japonica* produced in China is used from iodine extraction (FAO 2014b).

Protein-derived amino acids from macroalgae can also be a source of bio-based chemicals. Particularly glutamic acid has been recently utilized as precursor for the production of valuable chemicals such as N-methylpyrrolidone, N-vinylpyrrolidone, succinonitrile and acrylonitrile (Lammens et al. 2012). Although the protein content of *S. latissima* is relatively low, ranging seasonally from 1.3% to 10.8% DM, glutamic acid is the major amino acid, accounting up to 26% of the total amino acids (Paper II).

A number of carbohydrates have been recognized for their bioactivity including anticoagulant, antithrombotic, antiviral, immuno-inflammatory, antilipidemic and antioxidant, and thus have potential to be used for therapeutic applications. These include fucoidan and fucan (brown algae), galactan and ulvan (green algae), and carrageenan and galactan (red algae) (Holdt and Kraan 2011; Jung et al. 2013).

Carbohydrates from macroalgae can be used for the production of fuels and chemicals in a sugar-based biorefinery platform (Kraan 2013; Song et al. 2015).

Other high-value niche products that can be extracted from brown algae include bioactive compounds such as fucoxanthin and polyphenols (Bruton et al. 2009; Holdt and Kraan 2011).

Extraction of each of these products would have to be assessed on commercial terms and demonstrate the feasibility for pre-production of fuels and/or chemicals alongside the higher-value product, with particular attention to whether the scale of operation is appropriate (Bruton et al. 2009).

4.3 *Saccharina latissima* as novel feedstock for fermentation-based succinic acid production in a biorefinery approach

Macroalgae biorefinery aims at produce a number of products to maximize biomass utilization and reduce waste, and thus increase profitability. However, downstream processing involves different steps according to the physicochemical properties of the target compounds, with often different optimal condition for the different steps of the process, which presents a process integration challenge (Alvarado-Morales et al. 2009). Moreover, it is possible that some products can no longer be produced, or are produced at much lower yields, after extraction or bioconversion of other compounds, or simply are degraded during a previous step in the process flow (Paper IV). Thus the refinery facility should first define which products to produce and which sequence of unit operations to apply to achieve the highest profit-cascading approach (Alvarado-Morales et al. 2009).

In paper IV the potential of *S. latissima* to be used as feedstock for succinic acid production in a biorefinery context was investigated. Biomass harvested year-round was characterized for total sugars and total phenolic compounds (TPC), to evaluate its potential as feedstock for fermentation process and extraction of high added value products. Our results showed that *S. latissima* harvested in May-September should be most suitable as feedstock for a biorefinery facility. Moreover, both total sugars and TPC were higher for the biomass cultivated over two cultivations seasons compared to one cultivation season.

Enzymatic hydrolysis efficiency ranged from 70.0% to 98.7%, for *S. latissima* biomass harvested upon different harvest times, without pretreatment other than drying and milling. These results are comparable to thus reported by Alvarado-Morales et al. (2015) for *Laminaria digitata*

biomass (78.23%), and further confirm that harsh pretreatments (e.g. acid) are not required for efficient sugar recovery from *Laminaria*. Although in this study the enzymatic hydrolysis was performed for 48h, maximum concentration of fermentable sugars was achieved after 24h, and thus hydrolysis time could be shortened to reduce operational costs and to avoid risk of contamination- as observed in the 3-L-reactor experiment after 41 hours. Noteworthy, vat pasteurization of macroalga biomass at 70 °C for 15 minutes proved to be effective at preventing microbial contamination both during hydrolysis and fermentation processes.

A maximum succinic acid yield of 91.9% (g g^{-1} of fermentable sugars), corresponding to 70.5% of theoretical maximum, was achieved from a blend of macroalgae biomass cultivated over two growing seasons and harvested in July and August. This is one of the highest values reported in literature for *A. succinogenes* 130Z (Koutinas et al. 2014) and higher than that achieved from the fermentation of *L. digitata* hydrolysates (86.49%; g g^{-1} of total sugars) (Alvarado-Morales et al. 2015). Mannitol was utilized first followed by glucose, and both were depleted after 6 and 16 hours, respectively (Figure 9). Succinic acid titer reached 36.8 g L^{-1} with a maximum productivity of 3.9 g L^{-1} h^{-1}. Low by-product formation was observed; acetic-, formic acid and ethanol reached 7.9 g L^{-1} (20.0%), 1.4 g L^{-1} (3.5%), and 1.0 g L^{-1} (2.4%), respectively. Lactic acid formation was observed to a limited extent. The succinic acid titer achieved in this study is in the middle of reported values for *A. succinogenes* 130Z when using feedstocks such as cotton stalk hydrolysate, cheese whey, waste bread and wheat hydrolysate (15.8-64.2 g L^{-1}; Koutinas et al., 2014). Moreover, it is the highest titer ever reported when using macroalgae feedstock. Nevertheless, further research is needed to optimize macroalgae-based succinic acid production in order to meet the commercial target concentrations (120-150 g L^{-1}) (Urbance et al. 2004; McKinlay et al. 2007).

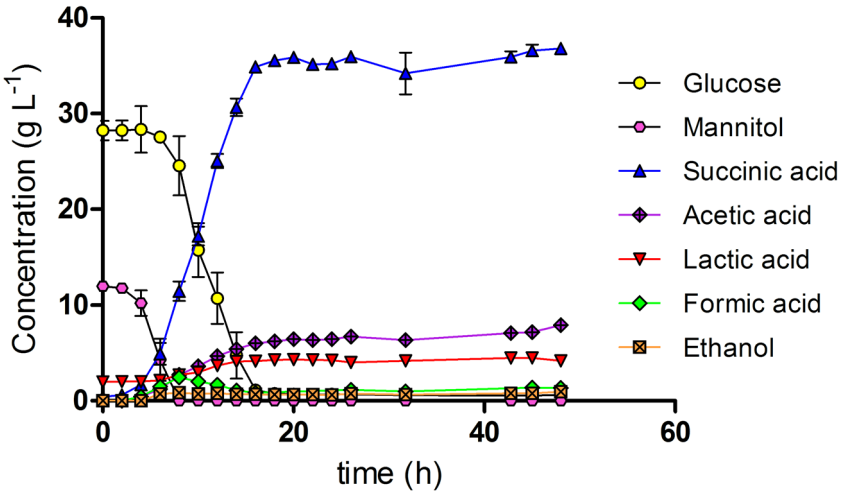


Figure 9. Fermentation profile for the 3-L-fermenter experiment.

The high concentration of TPC in the original macroalga, and macro- (Ca, K, Na, Mg, P, N and Fe) and micronutrients in the solid residue recovered after enzymatic hydrolysis, suggests that co-production of antioxidants (i.e. phenols) and fertilizers has high potential. Moreover, K, Na and Mg are estimated, based on the mass balance, to be present in the hydrolysate at concentrations of 3.00, 2.97 and 0.57 g L^{-1}, respectively (Supplementary data, Paper IV), and thus the addition of these nutrient to the synthetic fermentation media is likely unnecessary, which can result in a reduction in nutrient cost. Results further showed that the phenolic compounds have undergone degradation during enzymatic hydrolyses, and thus their extraction should be performed before enzymatic hydrolyses for a higher product recovery and revenue. This would require the use of green solvents (e.g. water, ionic liquids), to minimize the environmental impact and risk of microorganism inhibition during the subsequent fermentation process. On the other hand, this procedure could potentially result in the solubilisation of mannitol, which is easily extractable even with water (Horn et al. 2000a; Paper IV), leading to reduced amount of fermentable sugars in the subsequent steps- which requires further evaluation. The protein content, and quality, based on the content of essential amino acid such as lysine and methionine, of both the macroalga and the solid residue (recovered after enzymatic hydrolysis) were below the standards for common protein sources for fish feed (e.g. fish meal, soy meal), and thus were not considered as protein ingredient for fish feed. Moreover there was no significant difference in protein content and amino acid composition between the macroalga and the

solid residue recovered after hydrolysis. These results are opposite to those reported by Alvarado-Morales et al. (2015) who found that solubilisation of carbohydrates from *L. digitata* resulted in up-concentration of protein and lipids in the solid residue, 3.5 and 8.6 times, respectively, which improved its nutritional value. This can be explained by the extraordinary higher carbohydrate content of the macroalga used in that study (77.6% DM), and by the fact that the solid residue was washed with water to remove the remaining solubilised carbohydrates, resulting in further concentration of the other components-which was not done in the present study.

Finally, a simplified economic assessment (Paper IV) showed that at current macroalga feedstock price succinic acid production alone is negative for the whole range of succinic acid market prices (2.67 to 7.91 €/kg), while co-production of TPC and fertilizer turns the profit positive for mid-high range of succinic acid market price (above 4.77-6.87 €/kg SA) (Figure 10). Considering an advanced scenario, based on the expected reduction on the production cost of the feedstock in ten years' time (Holdt and Edwards 2014), succinic acid production alone would have a break-even point of 3.12 €/kg of succinic acid, while co-production (TPC, fertilizer and methane as well as CO_2 savings due to biogas upgrading and credits for CO_2 consumption) would result in a positive profit for the whole succinic acid market price.

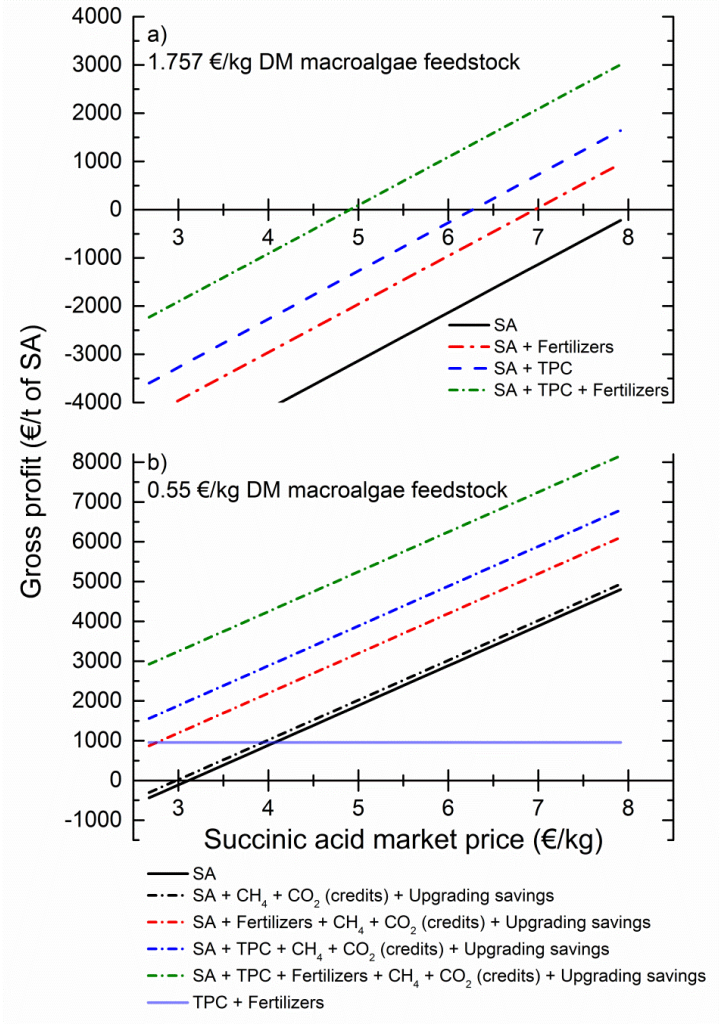


Figure 10. Gross profit (€/ton succinic acid-SA) for SA, and co-products considering; a) the current macroalga feedstock production cost of 1.757 €/kg DM and b) an estimated production cost in ten years' time of 0.55 €/kg DM.

4.4 Proposed biorefinery concept

Based on the findings on the biomass yield (Paper I), chemical composition (Paper II and IV; summarized in Figure 11) and potential of *Saccharina latissima* as feedstock for biorefinery with succinic acid and phenolic compounds as main products (Paper IV) a novel biorefinery concept is proposed (Figure 12). Furthermore, an operational plan is proposed for the macroalga cultivation (Figure 11).

S. latissima feedstock harvested from May to September would be preferable, since: 1) the content in fermentable sugars is higher (Paper IV), 2) the biomass yield is higher, and 3) the development of epiphytes in July-September makes this biomass unsuitable for human consumption (Paper I).

Moreover, cultivation over two growing seasons would further increase the concentration of fermentable sugars and TPC of the macroalga feedstock, making it most suitable for biorefinery (Paper IV). On the other hand, harvesting in November-February would result in significant lower biomass yield and concentration of fermentable sugars and thus is less suitable for biorefinery. Moreover, this also corresponds to the typical period of deployment of seeded lines at sea. Macroalga feedstock harvested in March-May could be used directly as food since this biomass is free of epiphytes and the yields are considerably higher than those found in November-February. However, considering potential restriction for human consumption, particularly regarding the high levels of iodine, this biomass may also be considered alternatively as feedstock for biorefinery.

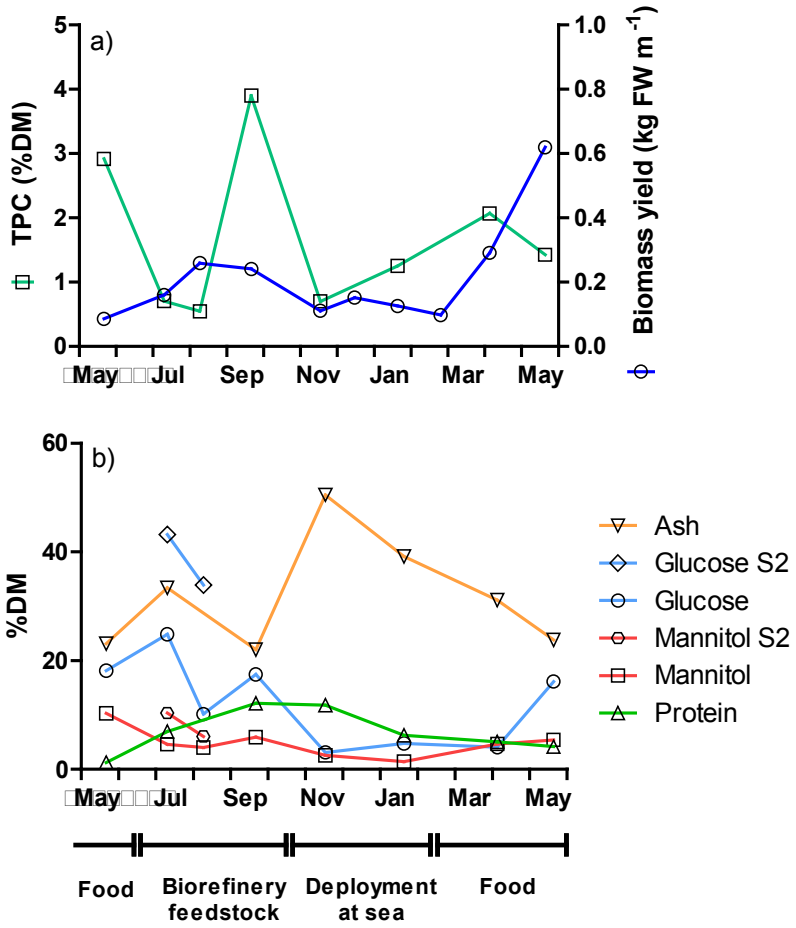


Figure 11. Seasonal variations in the concentration of total phenolic compounds (%DM) and biomass yield (a) and concentration of ash, protein, glucose and mannitol of *Saccharina latissima* (b). Glucose and mannitol concentration of macroalga cultivated over two growing season (S2).

Fresh or freeze-dried macroalgae rich in phenolic compounds would first be treated with a suitable solvent to obtain a phenol-rich extract after solvent recovery. This would avoid degradation of the phenolic compounds, as observed during the hydrolysis in Paper VI, and thus maximize product recovery and revenue. The leftover solid residue would then be enzymatically hydrolysed and after that the liquid fraction would be separated from the solid residue- which may require further optimization for up-scaling. The liquid fraction rich in fermentable sugars would be used for fermentation-based succinic acid production as demonstrated in Paper VI. After recovery of succinic acid from the fermentation broth the leftover effluent, which should contain considerable amounts of non-fermentable alginate, as well as solubilised protein and lipids, could be anaerobically digested to produce biogas. The solid residue recovered after enzymatic hydrolysis, containing remaining carbohydrates, protein and lipids could then be used to produce biogas through anaerobic digestion, or used directly as fertilizer, as it contains high concentrations of macro- and micronutrients, and important nutrients such as P, Ca and Fe were up-concentrated (Paper IV). The leftover digestate from anaerobic digestion, which should contain significant amount of leftover minerals, could also be used as fertilizer.

Moreover, there is potential for process integration with the CO_2 required for fermentation-based succinic acid production being supplied from biogas with simultaneous upgrading (red arrows in Figure 12) as demonstrated by Gunnarsson et al. (2014) or provided from other industries. This would result in environmental (i.e. CO_2 fixation) and economic (i.e. CO_2 credits, CO_2 savings, CH_4) benefits.

Finally, for the macroalga feedstock harvested in March-April, and considering potential restriction for human consumption, an alternative approach is proposed. Considering the lower concentration of fermentable sugars (i.e. glucose and mannitol), which in turn should be compensated with high concentration of alginate, fermentation-based succinic acid production does not seem a favourable process here. Thus first solvent extraction of the phenol-rich extract would be performed, to improve the cost-effectiveness of the process. This would be followed by anaerobic digestion of the solid residue to produce biogas to maximize carbon utilization/conversion. The leftover digestate could be used as a mineral-rich macroalga fertilizer.

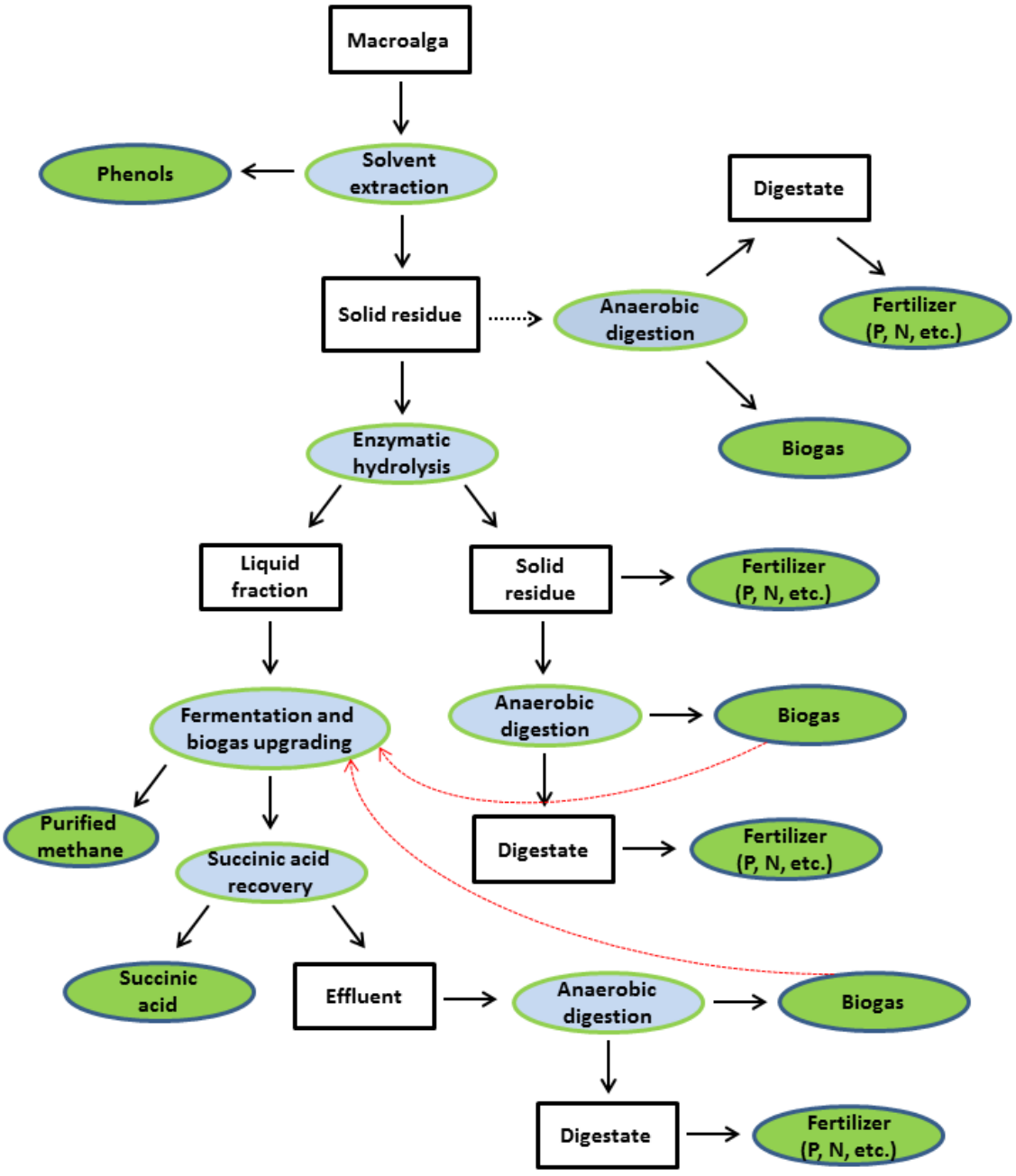


Figure 12. Flow diagram of the biorefinery concept.

5 Conclusions

This thesis focused at optimizing the bioremediation capacity of macroalga *Saccharina latissima* targeting the recovery of N and P, considering harvest time, biomass yield and N and P content. Moreover, biomass valorisation was performed through the evaluation of its chemical composition and nutritional value for especially human food and animal feed, and testing its potential as feedstock for biorefinery. The major contribution resulting from these studies are summarized below.

- Harvest time can be effectively used to optimize the bioremediation capacity (N and P removal), and the biomass yield and application/value.
- An estimated maximum areal yield of 7.1 t FW ha^{-1} could be achieved by harvesting the biomass in September (one growing season) and May (two growing seasons), corresponding to a N removal of 39.4 and 26 kg ha^{-1}, respectively.
- Changes in the concentrations of N and P in the macroalga were driven by seasonal variations in the ambient nutrient background concentrations, while nutrient input from the fish farm was apparently negligible.
- Overall chemical composition and nutritional value changed seasonally, while cultivation site did not generally explain significant changes.
- Both concentrations of protein and lipids peaked in November, while biomass for human consumption is harvested in May. These results suggest an apparent mismatch between harvest time and the highest nutritional value.
- The macroalga can be a source of valuable amino acids especially as human food. However, the protein content and amino acid composition do not comply with the requirements for standard protein ingredients for fish feed. Moreover, the high concentrations of iodine and total arsenic may be of concern regarding safety regulation for food and feed, respectively.
- The macroalga biomass also including epiphytes is a suitable feedstock for fermentation-based succinic acid production, and co-production of phenols and fertilizers. Maximum succinic acid yield of 91.9% (g g^{-1} of total sugars) was achieved, while succinic acid titer amounted up to 36.8 g L^{-1}.

- Seasonal changes in the chemical composition of the macroalga feedstock will have a dramatic impact in the biorefinery facility, which has been addressed in a proposed biorefinery concept.

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In this online version of the thesis, **paper I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four sections:
Water Resources Engineering, Urban Water Engineering,
Residual Resource Engineering and Environmental Chemistry & Microbiology.

The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

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